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学位授与の題目	In vitro and in vivo toxicological evaluation of acyl glucuronides （ In vitro および in vivo におけるアシルグルクロナイドの毒性学的評価）
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学位論文要旨

Abstract

Glucuronidation is an important phase II metabolic pathway for endogenous and exogenous substrates and is generally considered as a detoxification pathway, but acyl glucuronides (AGs) have been believed to be related with the toxicity of carboxyl acid drugs, because AGs covalently bind to endogenous proteins owing to their instability. However, the theory remains controversial. In this study, first, *in vitro* assays of half-lives, peptide adducts and immunostimulation of AGs of 21 drugs were performed, and the relationship to toxic categories of drugs was analyzed. Short half-lives, peptide adducts and immunostimulation were observed in AGs of all withdrawn drugs tested in this study. The immunostimulation assay showed higher sensitivity, specificity, and accuracy than half-lives and peptide adducts assays. Second, responsibility of zomepirac acyl glucuronide (ZP-AG) for renal toxicity by zomepirac (ZP) was investigated by *in vivo* studies. ZP-induced kidney injury mouse model was established by pretreatment with an esterase inhibitor and a glutathione synthesis inhibitor. It was demonstrated that ZP-AG accumulation in kidney was responsible for the renal toxicity. This study provides new insight into the evaluation of AG toxicity in drug development.

Dissertation abstract

Glucuronidation, an important phase II metabolic pathway, is generally considered as a detoxification pathway, but AGs of drugs are generally unstable and are believed to be involved in drug-induced toxicity via the formation of covalent adducts to endogenous proteins. However, the toxicity of AGs remains unclear. This study had two purposes. First purpose was to evaluate the toxicity of AGs by utilizing the *in vitro* assays of half-lives, peptide adducts and immunostimulation. ZP is one of carboxyl acid drugs withdrawn from the market due to adverse effects such as renal toxicity and anaphylaxis. It was demonstrated, by previous *in vitro* studies, that ZP-AG formed protein adducts, suggesting that ZP-AG was involved in toxicity caused by ZP. However, the toxicity of ZP-AG has not been proved by *in*

vivo study. Second purpose was to investigate by *in vivo* study whether ZP-AG is responsible for renal toxicity by ZP.

In vitro toxicological evaluation of acyl glucuronides by half-lives, peptide adducts, and immunostimulation assays

Chemical reactivity of AGs is believed to be involved in the toxicity of carboxylic acid-containing drugs. Both direct and immune-mediated toxicity have been suggested as possible mechanisms of toxicity; however, it remains unclear. In this study, the assays of half-lives, peptide adducts, and immunostimulation were performed to evaluate the potential risk of AGs of 21 drugs and analyzed the relationship to the toxic category. AGs of all withdrawn drugs tested in this study showed short half-lives and peptide adducts formation, but so did those of several safe drugs. In contrast, only AGs of withdrawn and warning drugs induced IL-8 in hPBMCs. It was found by a DNA microarray assay that zomepirac AG induced the mRNAs of 5 genes, including IL-8 in hPBMCs. In addition, withdrawn and warning drugs were distinguished from safe drugs by an integrated score of relative mRNA expression levels of 5 genes. The immunostimulation assay showed higher sensitivity, specificity, and accuracy compared with other methods. In preclinical stage in drug development, the evaluation of immunostimulation by AGs using hPBMCs can contribute to improved drug safety assessment.

In vivo toxicological evaluation of zomepirac acyl glucuronide

Zomepirac (ZP) was withdrawn from the market because of their adverse effects such as renal toxicity. ZP is mainly metabolized to acyl glucuronide (ZP-AG) by UGT. However, the responsibility of ZP-AG to renal toxicity has never been proven. In this study, ZP-induced kidney injury mouse model was established by pretreatment with tri-*o*-tolyl phosphate (TOTP), a non-selective esterase inhibitor, and L-buthionine-(*S*,*R*)-sulfoximine (BSO), a glutathione synthesis inhibitor, and then the responsibility of ZP-AG to renal toxicity was investigated. Mice received only ZP showed significant increase of blood urea nitrogen (BUN) and creatinine (CRE) by pretreatment with TOTP and BSO, but not increase of alanine aminotransferase. The ZP-AG levels in plasma and liver were elevated by co-treated

with TOTP, and especially in kidney. The ZP-AG concentrations in kidney were correlated with BUN and CRE. In histopathological examination, the vacuoles and infiltration of mononuclear cells were observed in mouse model. In addition to immune- and inflammation-related responses, the oxidative stress markers such as glutathione/disulfide glutathione ratio and malondialdehyde (MDA) levels were changed by TOTP, BSO and ZP-administration. Since the ZP-induced kidney injury was suppressed by treatment with tempol, an antioxidant agent, the involvement of oxidative stress was suggested in the ZP-induced kidney injury. This is the first study to demonstrate that AG accumulation in kidney by TOTP and BSO-treatment was responsible for the renal toxicity and to show the *in vivo* toxicological potential of AGs.

References:

1. Iwamura A, Ito M, Mitsui H, Hasegawa J, Kosaka K, Kino I, Tsuda M, Nakajima M, Yokoi T, Kume T. (2015) Toxicological evaluation of acyl glucuronides utilizing half-lives, peptide adducts, and immunostimulation assays. *Toxicol In Vitro* **30**:241-249.

審査結果の要旨

アシルグルクロナイド（AG）はその反応性の高さからタンパク質への共有結合を介して毒性発現に関与することが示唆されているものの、詳細については不明な点が多い。本研究は、in vitro および in vivo で AG について毒性学的に評価することを目的としたものである。In vitro 評価では、21 薬物の AG について半減期法、ペプチドアダクト法および免疫活性化法による評価を行った。半減期法およびペプチドアダクト法では撤退薬および警告薬のみならず安全薬についても短半減期およびペプチドアダクトが検出された。免疫活性化法では、撤退薬および警告薬においてのみ活性化作用が認められ、3 法の中で最も高い判定精度を示した。In vitro 評価において最も毒性ポテンシャルの高かった撤退薬ゾメピラクについて AG 体の in vivo 毒性を評価した。加水分解酵素阻害剤およびグルタチオン枯渇剤を併用投与することで、臨床における主要な副作用である腎障害を発症するモデルマウスの作製に成功した。本モデルマウスにおいて、腎組織中に AG が高濃度に蓄積していることから AG 体が腎障害を誘発している可能性が高いことを示した。以上、本研究成果は免疫活性化法の in vitro AG 毒性評価法としての有用性および in vivo レベルでの AG の毒性への関与を示し、カルボン酸化合物の安全性評価に貢献するものであり、博士（創薬科学）に値すると判定した。